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Dimethylacetamide - an alternative to glycerol as cryoprotectant of Malabari buck semen#

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Abstract

The key role of a cryoprotectant is to minimize the chemical and physical stress which occurs during cooling, freezing and thawing of semen. The difference between the cryoprotectant (CPA) occurs in their permeability coefficient and the structural model of the cryogenic agent. The beneficial effect of dimethylacetamide (DMA) as a cryoprotectant especially for sperms had been observed in several studies. The aim of the study was to study the cryoprotective effect of DMA in freezing the Malabari buck semen compared to glycerol. Ten ejaculates were taken from fourMalabaribucks . After preliminary evaluation sample split technique was followed with Tris based extender containing glycerol (6.7 per cent) as cryoprotectant (control) and Trisextender containing DMA (3 per cent) as cryoprotectant (treatment group). The semen straws (0.25mL) after filling were subjected for equilibration and manual freezing. Sperm kinetics was studied using computer-aided sperm analyzer. Pre-freeze and post-thaw evaluation included sperm viability, sperm abnormality, hypo osmotic test, acrosome integrity test and DNA fragmentation. Results indicated that inclusion of 6.7 per cent glycerol had significantly higher ($p<0.05$) post-thaw values than DMA. From our study we conclude that 6.7 per cent glycerol was better than 3 per cent DMA in cryopreservation of Malabari buck semen.

Keywords: *Cryopreservation, dimethylacetamide, glycerol*

Success of cryopreservation depends on not only preserving motility and viability of the spermatozoa but also in retaining the fertilizing capacity of the sperm. Tolerance of sperm to different components of the diluents differs between the species, breeds and individual animals. For long period of time glycerol has been considered an ideal cryoprotectant for cryopreservation of buck semen. But recent studies revealed the lethal effects of glycerol to the sperm membrane. Amide group of cryoprotectant (CPA) serves as an alternative to glycerol in many species (stallion,

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crane, kestrel etc.) (Vidament*et al*., 2009). Theoretically, amide group of cryoprotectants have low viscosity and molecular weight. Effective cryoprotectant property of amides comes from possessing long pairs of electrons (Hwang *et al.,* 2009). Amide reduces the viscosity in the semen diluent compared to glycerol (Alvarenga*et al*., 2005). The present study has been conducted to reveal the cryoprotective efficacy of DMA over glycerol in reducing cryo-injury and enhancing the post-thaw quality of Malabari buck semen.

Material and methods

Four adult healthy Malabari bucks aged between two and four years, maintained at the Artificial Insemination Centre, Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Pookode, Wayanad have been utilized for the study. Semen ejaculates were collected from four Malabari bucks twice weekly at an interval of three days between collections using Danish type artificial vagina which was maintained at 42-45ºC under adequate pressure.

Fresh semen evaluation

Preliminary evaluation included assessing the volume, density, colour and mass activity. After initial dilution, individual motility was assessed using phase contrast microscope (400 x). As the buck semen is highly concentrated, a higher dilution rate (1:1000 dilution) was considered for precise calculation of sperm concentration using Neubauer counting chamber. Sperm viability was assessed using eosin-nigrosin staining technique. The percentage of sperm abnormalities was recorded from the smear prepared for assessing viability under 1000 x objective of the bright field microscope. A minimum of 200 sperms were counted from different microscopic fields. Before freezing pre-freeze evaluation was carried out. Sperm motility, sperm viability and sperm abnormality were carried out as mentioned for fresh semen evaluation.

HOS-G test

Pre-freeze and post-thaw membrane integrity were assessed using hypo osmotic swelling- Giemsa test (HOS-G) in which sperms were subjected to hypo osmolar solution (100 mOsm) (Trisodium citrate dihydrate: 0.49g; Fructose:0.99g) was compared with control solution (300mOsm) (Trisodium citrate dihydrate:1.47g; Fructose: 2.97g). After incubation for 30 minutes at 37˚C, smears were prepared and immersed in buffered formal saline (BFS) for 30 minutes. The slides were washed in tap water and dried. The dried slides were subjected for overnight Giemsa stain at 37˚C. A minimum of 200 sperms were counted with 400x magnification (Carl Zeiss Axiostar, Germany) for assessing functional membrane integrity and acrosome integrity (Selvaraju*et al*., 2008).

DNA integrity assessment using acridine orange stain

DNA integrity was assessed using acridine orange (AO) stain (Sigma-Aldrich®,USA). Each semen sample was centrifuged at 3000 rpm, 4˚C for 5 min. The sperm pellets werewashed by adding 2 ml of PBS (pH – 7.2). After centrifugation the sperm pellets werere-suspended in PBS. A smear was prepared from the sperm suspension and air dried on a glass slide. The smear was fixed in Carnoy's fixative for two hours, rinsed and air dried. The smear was stained with AO stain solution for 15 mins. After the staining, smear was washed thrice with PBS, air dried and examined immediately under a fluorescent microscope (Zeiss Axiolab Fluorescent Digital microscope) (excitation of 450 – 490nm). Spermatozoa with yellow, orange or red fluorescence indicated DNA damage whereas green fluorescence indicated intact DNA. A minimum of 200 sperms were counted from different microscopic fields and percentage of spermatozoa with double and single stranded DNA were calculated based on green and red or yellow fluorescence stained spermatozoa respectively (Chohan*et al.,* 2004).

Semen processing for cryopreservation

Semen samples with ++++ mass activity and 70 per cent progressive motility were considered for cryopreservation. One step addition of the extender to the semen samples was carried out based on progressive motility and sperm concentration. The prepared TRIS extender was divided into two groups. The control group was added with 6.7 per cent glycerol whereas the treatment group contained 3 per cent DMA. The extended semen was filled in 0.25 mL sterile French mini straws manually. Manual sealing was carried out using polyvinyl alcohol powder. Before the onset of freezing, the straws were chilled to 5˚C for two hours and equilibrated for two hours for control group and one hour for treatment group in cold holding cabinet (IMV technologies®, France). Manual freezing was carried out by placing the straws horizontally at four centimeters above the level of liquid nitrogen for 10 minutes and then immersed into the liquid nitrogen. The straws were collected into a goblet and plunged into liquid nitrogen in a cryocan for storage (Shi*et al*., 2014).

Post-thaw evaluation

One straw from each group was taken from the cryocan. After (37˚C for 30 sec) samples were emptied into separate micro centrifuge (Tarson, India) tubes kept at 37˚C in a water bath. The sperm kinematic parameters were analyzed using a computer-aided sperm analyzer (CASA; Sperm Class Analyser, Version 6.4, Microptic SL, Spain) with parameters as in Table 1. On a clean prewarmed microscopic slide, 10 µL of sample was placed and covered by a coverslip on the microscopic stage maintained at 37˚C with a stage warmer. The analysis was performed by capturing minimum of 10 random homogenous fields using a camera (Nikon eclipse 50 Nikon, Japan) with a capturing efficiency of 25 frames/s at 10 x magnification in the negative phase-contrast microscope (Nikon eclipse 50, Nikon, Japan). Egg yolk particles and debris present in the captured fields were manually removed to eliminate them from the analysis. The particle size within the range of 3-70µ would be estimated. The curvilinear velocity was used to classify the progressive motility of the spermatozoa into rapid, medium, slow and static. The following CASA settings were adopted for sperm tracking.

Table 2. Settings in computer assisted sperm analysis for the assessment of sperm kinematics

CASA parameters	Criteria set for analysis
Particle size	3-70µ
Motility and Progressive (%)	Rapid: VCL > 75 µm/s
	Medium: VCL > 40 µm/s
	Slow: VCL - 10 – 25 µm/s
	Static: VCL > 10 µm/s
	Progressive: STR >80

Result and discussion

Sperm motility

Control group provided higher pre-freeze motility (74.25 ± 0.73 per cent) comparing to the treatment group (66.0 ± 1.75 per cent). Post-thaw motility of the control group (48.05 ±5.84 per cent) was significantly ($p<0.05$) high compared to the treatment group (32.19 ± 3.28 per cent). Progressive motility of the sperm could be highly correlated with the fertilizing capacity of the sperm (Bencharif *et al*., 2013). Anakkul *et al*. (2013) reported 7 per cent glycerol included in Black Bengal buck sperm freezing was not suitable for producing required cellular dehydration at the time of freezing which resulted in poor post-thaw motility (30.9 per cent). On the contrary, in the present study increased post-thaw total motility (48.6 per cent) was obtained in Malabari buck.

Bittencourt *et al*. (2018) obtained total post-thaw motility of 60.6 ± 14.2 per cent with 3 per cent DMA which was significantly high ($p<0.05$) compared to the present study (32.19 ± 3.28 per cent). Inclusion of higher concentration of DMA as cryoprotectant in other species like stallion (5 per cent) (Alvarenga *et al*., 2005), kestrel (12.3 per cent) (Brock and bird,1991), crane (26 per cent) (Blanco *et al*., 2011) produced better results compared to the present study.

Krishnan *et al*. (2018) obtained 43.33 ± 1.05 per cent post thaw spermatozoa motility in Malabari buck semen with glycerol as cryoprotectant which was lesser than the present study.

Sperm motion characteristics are very sensitive to physical and chemical components of the semen extender. Osmolarity of the semen extender influences the motion characteristics. Seifi-jamadi *et al*. (2017) recorded that straightness (STR) and linearity (LIN) parameters were significantly high ($p<0.05$) in buck spermatozoa cryopreserved with DMA compared to glycerol, whereas in present finding treatment group did not show any positive correlation with sperm kinetics. Sperm kinetics did not vary significantly($p>0.05$) between the groups.

Sperm viability and abnormality

Pre-freeze sperm viability did not show any significant difference ($p>0.05$) in pre-freeze sperm viability whereas post-thaw sperm viability showed significant difference ($p<0.01$) between the groups. Control group (48.25 ± 0.75 per cent) showed increased post-thaw viability than the treatment group (35.13 ± 1.17 per cent). No significant difference ($p>0.05$) could be noticed in sperm abnormality between groups in both pre-freeze and post-thaw evaluation.

Present findings are discordant with Seifi-jamadi *et al*. (2017) who observed higher post-thaw sperm viability (53.70 ± 1.45 per cent) when compared to glycerol (50.35 ± 1.07 per cent) in buck semen. The cryoprotective effect of a CPA was greatly influenced by equilibration time and concentration of CPA used (Deka and Rao, 1986; Iaffaldano *et al*., 2012). In a study, semen exposure time was kept as 45 minutes for cryopreservation of rabbit semen (Iaffaldano *et al*., 2012) which was comparatively less than the current study (1 hour), but reduced viability (23.16 ± 1.90 per cent) was noticed when compared to the present study (35.13 ± 1.17 per cent). Difference in composition of phospholipid layer in the sperm membrane of each species governs difference in permeability coefficient of CPA agent.

Neville *et al*. (1971) observed sperms frozen with DMA had longer viability in female genital tract which resulted in increased pregnancy rate comparing to glycerol (46%). Pregnancy rate was less in mares inseminated with spermatozoa cryopreserved with glycerol (Moffet *et al*., 2003) and ewes (Bittencourt *et al*., 2018). This could be related to the contraceptive property of glycerol in certain species. Kim *et al*. (2011) observed higher per cent of sperm morphological defect with 3 per cent DMA in boar spermatozoa. These findings were consistent with observations of the present study that spermatozoa cryopreserved with 3 per cent DMA revealed higher morphological defects compared tothose cryopreserved in 6.7 per cent glycerol.

Functional membrane integrity and acrosome integrity

Pre-freeze membrane integrity evaluation did not show any significant difference ($p>0.05$) between the groups in pre-freeze evaluation. The pre-freeze membrane integrity of the control and treatment groups were 68.75 ± 1.31 and 65.88 ± 0.88 respectively (Table 3). In post-thaw assessment, control group showed significantly ($p<0.05$) higher functional membrane integrity (53.00 ±1.70 per cent) and acrosome integrity (55.75 ±1.00 per cent) compared to treatment group in which functional membrane integrity was 41.25 ± 1.52 per cent and acrosome integrity was 43.00 ± 1.17 per cent (Table 4).

Structural difference exists in the plasma membrane of the sperm among the species (Ladha, 1998). CPA with different chemical groups has different permeability coefficient in different species (Menezes *et al*., 2021).Kim *et al*.(2011) observed lower acrosome integrity with 3 per cent DMA compared to glycerol. Bittencourt *et al*. (2018) did not observe any significant difference between 6 per cent glycerol and 3 per cent DMA with respect to membrane integrity. On the contrary, in the present study, control group with 6.7 per cent glycerol provided better membrane integrity compared to 3 per cent DMA. Several authors (Bianchi *et al*., 2008; Seifi-jamadi e*t al*., 2017) recorded significantly higher post-thaw membrane integrity with DMA when compared with glycerol. These findings were discordant with present study. In the current study, the treatment group recorded the least functional membrane integrity and acrosome integrity.

Deka and Rao (1986) observed lower concentration of glycerol (4 per cent) with one hour equilibration time resulted in relatively lesser acrosomal damage than the other concentrations of glycerol (7 and 9 per cent) and equilibration time (3hrs and 5 hrs). They concluded that semen exposure time to CPA plays crucial role in obtaining better post-thaw semen variables.

Some authors recorded that the deleterious effect of amide was due to higher permeability via plasma membrane into the sperm cell, which resulted in increased concentration of DMA inside the cell. This could lead to development of DMA toxicity and high level of cellular dehydration at the time of freezing process. Excess cell dehydration could damage the functional membrane and organelles of the sperm cell (Menezes *et al*., 2020). This finding is corroborated with the present finding that 3 per cent DMA has destabilized the buck sperm membrane.

DNA integrity

The pre-freeze DNA fragmentation was 1.63 ± 0.26 and 1.75 ± 0.25 per cent of control and treatment group respectively (Table 3). The mean ± SE of post-thaw DNA fragmentation index of control and treatment groups were 2.38±0.26 and 2.38± 0.18 respectively (table 4). DNA strand breaks and base oxidization are promoted during freezing–thawing have been described in several species. Hwang *et al*. (2009) stated amide group (dimethylformamide) had favourable effect over maintaining the DNA integrity, whereas Gliozzi *et al*. (2011) recorded no significant difference between same concentration of glycerol and DMA over DNA integrity. Our findings are consistent with the latter finding of presence of either glycerol or DMA did not reveal any significant difference in preserving DNA integrity.

Conclusion

In conclusion, the present study indicated that post-thaw semen variables were comparatively well preserved with glycerol (6.7 per cent) than 3 per cent DMA. Despite low molecular weight, high permeability and better post-thaw sperm parameters were recorded in other species (equine, crane and kestrel), but

Table 3. The effect of glycerol and DMA on pre-freeze semen variables of Malabari buck sperm

Parameters	Control	Treatment	T-Value (p value)
Sperm motility	74.25 ± 0.73	66.0 ± 1.75	4.349* (p<0.05)
Sperm viability	72.13 ± 1.47	70.13 ± 1.32	1.014^{ns} (0.328)
Sperm abnormality	4 ± 0.46	4.25 ± 0.45	-0.386^{ns} (0.7)
HOST test	68.75 ± 1.31	65.88 ± 0.88	1.829^{ns} (0.08)
Acrosome integrity	68.13 ± 1.77	63.88 ±1.41	1.881^{ns} (0.08)
DNA integrity	1.63 ± 0.26	1.75 ± 0.25	-0.344^{ns} (0.73)

Table 4. The effect of glycerol and DMA on post-freeze semen variables of Malabari buck semen

Parameters	Control (%)	Treatment	T-Value (p value)
Sperm motility	48.05 ± 5.84	32.19 ± 3.28	2.367* (0.03)
Sperm viability	48.25 ± 0.75	35.13 ± 1.17	9.435** (0.000)
Sperm abnormality	7.25 ± 0.45	7.63 ± 0.38	0.638^{ns} (0.5)
HOST test	53.00±1.70	41.25±1.52	2.335* (0.03)
Acrosome integrity	55.75 ±1.00	43.00 ± 1.17	10.249** (0.000)
DNA integrity	2.38±0.26	2.38± 0.18	0.000 (1.00)

* Significant at 0.05 level

** Significant at 0.01 level

^{ns} Non significant

Independent t test - Between the group (Control and treatment group)

no such amelioration noticed with DMA in the present study. Further studies are required to standardise the concentration and equilibration time of DMA in freezing Malabari buck sperm.

Conflict of interest

The authors declare that they have no conflict of interest.

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